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A conserved and species-specific functional interaction between the Werner syndrome-like exonuclease *at*WEX and the Ku heterodimer in *Arabidopsis*

Baomin Li, Nathan Conway[1], Sonia Navarro, Luca Comai[1] and Lucio Comai*

Department of Molecular Microbiology and Immunology, Keck School of Medicine, University of Southern California, LA, USA and [1]Department of Biology, University of Washington, Seattle, WA, USA

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ABSTRACT

Werner syndrome is associated with mutations in the DNA helicase RecQ3 [a.k.a. *Homo sapiens* (*hs*)-WRN]. The function of *hs*WRN is unknown although biochemical studies suggest a role in DNA ends stability and repair. Unlike other RecQ family members, *hs*WRN possesses an N-terminal domain with exonuclease activity, which is stimulated by interaction with the Ku heterodimer. While this interaction is intriguing, we do not know whether it is important for *hs*WRN function. Although flies, worms, fungi and plants do not have RecQ-like (RQL) helicases with an intrinsic exonuclease activity, they possess proteins having domains homologous to the *hs*WRN exonuclease. The genome of *Arabidopsis thaliana* (*at*) encodes multiple RQL and a single protein with homology to the WRN exonuclease domain, *at*WEX (Werner-like Exonuclease). Here we show that *at*WEX has properties that are similar to *hs*WRN. *at*WEX binds to and is stimulated by *at*Ku. Interestingly, stimulation by Ku is species-specific, as *hs*Ku does not stimulate *at*WEX exonuclease activity. Likewise, *at*Ku fails to enhance the exonuclease activity of *hs*WRN. Thus, in spite of the differences in structural organization, the functional interaction between WRN-like exonucleases and Ku has been preserved through evolutionary radiation of species, emphasizing the importance of this interaction in cell function.

INTRODUCTION

Werner syndrome (WS), a human disease with many features of premature aging, is caused by mutations within a single gene located on human chromosome 8. The disease becomes evident in late adolescence and involves the frequent occurrence of conditions generally observed during normal aging, such as atheriosclerosis, osteoporosis, type II diabetes mellitus, myocardial infarction and cancer (1). Cells isolated from WS patients display a shorter replicative life span (2) and genomic instability characterized by an elevated rate of chromosomal translocations and extensive genomic deletions (3). The gene mutated in WS encodes a protein [*Homo sapiens* (*hs*)RecQ3 or WS protein—*hs*WRN] that is a member of the RecQ family of helicases (4). In contrast to other RecQ helicases, *hs*WRN possesses an N-terminal domain with exonuclease activity. The presence of exonuclease and helicase activities suggests that WRN is involved in a nucleic acid reaction; however, the precise cellular function of this protein remains poorly defined. During the last few years, a growing number of biochemical studies have reported the identification of proteins that interact with *hs*WRN (5–7). Work from our and another laboratory has shown that *hs*WRN binds to the Ku70/80 heterodimer (Ku) (8,9). Remarkably, our studies showed that Ku recruits *hs*WRN to DNA ends and alters the properties of *hs*WRN exonuclease activity (8,10). Ku is a factor that binds to DNA ends and is involved in the repair of double-strand DNA breaks by non-homologous DNA end joining (NHEJ) (11). Moreover, Ku is also found at telomeric ends (12), suggesting that this factor may, depending on the context, stimulate or inhibit ligation of DNA ends. How these distinct functions are regulated is currently unknown, but it is possible that specific protein interactions play a role in

*To whom correspondence should be addressed. Tel: +1 323 442 3950; Fax: +1 323 442 2764; Email: comai@usc.edu
Correspondence may also be addressed to Luca Comai. Tel: +1 206 543 4841; Fax: +1 206 685 1728; Email: comai@u.washington.edu

The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors

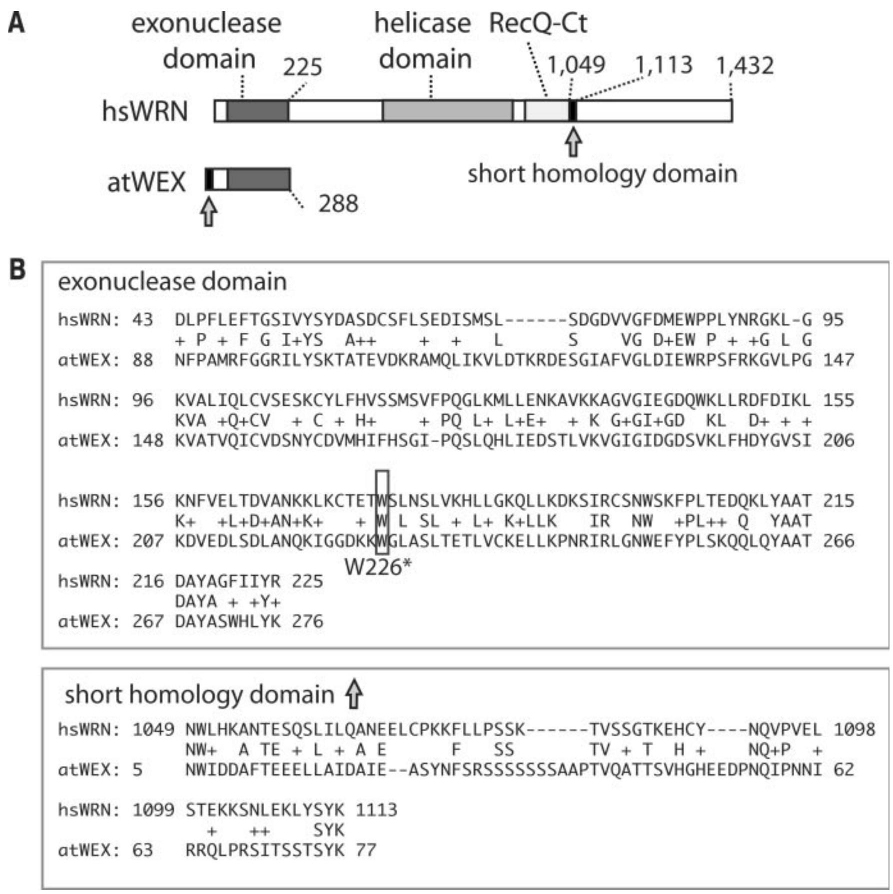

Figure 1. Domain organization of *hs*WRN and *at*WEX. Structural comparison of hsWRN and atWEX. (**A**) Schematic map of conserved domains in hsWRN and atWEX. The arrow points to a short region of homology outside the exonuclease domain that is rearranged between the two proteins. (**B**) Sequence alignments between hsWRN and atWEX showing the exonuclease domain and the short homology domain. The truncation in the predicted product of the *wex*-2 allele is marked (W266STOP). RecQ-Ct = RecQ C-terminal domain (amino acids 949–1092).

determining the fate of DNA ends bound to Ku. It remains to be determined whether the interaction between *hs*WRN and Ku is important for some aspects of DNA ends metabolism or possibly other cellular processes. One way to assess the biological relevance of the interaction between these proteins is to test whether it is conserved across phylogenetically distant taxa. While the bifunctional structure of *hs*WRN with both an exonuclease and a helicase is conserved between human and mice, there are no known RecQ-like (RQL) helicases with an intrinsic exonuclease activity in flies, worms, fungi or plants. Nevertheless, genes in the *Arabidopsis* genome encode two types of proteins with high homology to WS protein: multiple RQL and a single exoC-domain exonuclease (Werner-like Exonuclease, *at*WEX) (13) (Figure 1). Biochemical studies have shown that *at*WEX has 3′–5′ exonuclease activity (14) and yeast-two-hybrid assays have indicated that it binds to RQL2, which among the RQLs is phylogenetically closer to hsWRN (13). Moreover, a study of an *Arabidopsis* T-DNA insertion mutant line with reduced *at*WEX mRNA expression showed that *at*WEX is required for post-transcriptional gene silencing (15), an epigenetic mechanism of gene expression regulation related to RNA interference. Interestingly, *at*WEX is homologous to *Caenorhabditis elegans* mut7; genetic analysis of mutated worm strains indicated that mut7 gene product is required for transposon silencing and RNA interference (16). However, the analysis of mice with a null mutation in the WRN homologue gene failed to show any involvement of this protein in the RNA interference pathway (17).

In this study, we characterize the properties of *at*WEX and examine its relationship to *at*Ku. We demonstrate that *at*WEX physically interacts with *at*Ku and *hs*Ku. Moreover, we present data showing that *at*WEX exonuclease activity is stimulated by *at*Ku but not *hs*Ku and, similarly, *hs*WRN exonuclease is stimulated by *hs*Ku but not by its plant counterpart. Thus, we conclude that Ku–WRN interaction is conserved and specific.

MATERIALS AND METHODS

Cloning of atWEX, atKu70 and atKu80

RNA was extracted using the Trizol method (Invitrogen) from flower buds of *Arabidopsis thaliana* accession Columbia (Col-0) *er105* for wild-type *atWEX*, from the mutagenized derivative of Col-0 *er105* for *wex-2*, and from Col-0 (for *at*Ku70 and *at*Ku80). A first strand cDNA segment containing the complete open reading frame of the target genes was synthesized by RT–PCR using RT–MMLV and random hexamers according to established protocols. The following primers for each gene were designed with NdeI sites (underlined) adjacent to the start ATG using Primer3 and then used to amplify the cDNA with Klentaq LA (Clontech). *atWEX*, 5′-CCATATGTCATCGTCAAATTGGATCGACGAC-3′ and 5′-TGAGCCACTGACAGCATCAGGAA-3′; *atKu70*, 5′-CATATGGAATTGGACCCAGATGATG-3′ and 5′-CCAGTTCCCATCAAAAACAGACAA-3′; *atKu80*, 5′-CATATGGCACGAAATCGGGAGGGTTT-3′ and 5′-TTGTTAGCTCTCGAGCATTGACTCTTGT-3′. PCR products were cloned using the TOPO TA cloning kit (Invitrogen). Plasmid DNA was then prepared using a plasmid extraction kit (Qiagen) and sequenced by the Big-Dye fluorescent chain-terminator method. The sequence was analyzed using Sequencher (v4.1.2). The *wex*-2 allele (W226*) of the *atWEX* gene (At4g13870) was identified in a TILLING screen (18). This mutation disrupts a restriction site for the enzyme NlaIV, facilitating genotyping.

Expression and purification of recombinant proteins

Flag-tagged *at* WEX, HA-*at* Ku80, His-*at* Ku80, myc-*at* Ku70 and *at* Ku70 were expressed individually or in various combinations in sf9 cells using a baculovirus expression system. The cDNAs coding for these factors were cloned into a pVL-based vector, and were then cotransfected with linearized BaculoGold DNA (Pharmingen) into *sf9* cells to generate the recombinant baculoviruses. Cells infected with the recombinant baculovirus expressing FlagWEX were lysed in Lysis Buffer (10 mM Tris, pH7.9, 100 mM KCl, 1.5 mM $MgCl_2$ and 0.7% Nonidet P-40) and FlagWEX was purified by chromatography on anti-Flag M2 agarose (Sigma). Cells infected with two recombinant baculoviruses expressing His-*at* Ku80 and *at* Ku70 were lysed in NTN buffer (0.7% NP-40, 20 mM Tris, pH 8 and 100 mM NaCl) and the *at* Ku complex was then purified by chromatography on a nickel-sepharose column (Pharmacia). All the buffers were supplemented with a cocktail of protease inhibitors [1 mM phenylmethlysulfonyl fluoride (PMSF), 1 mg/ml pepstatin A, 5 mg/ml leupeptin and 5mg/ml aprotinin]. Baculovirus amplification and *sf9* cells were maintained as described in (19). Recombinant *hs*WRN and Ku were purified from baculovirus-infected *sf9* cells as described previously (8).

Protein interaction assays

Sf9 cells infected with the appropriate recombinant baculoviruses were harvested 42–48 h postinfection and washed with 1× phosphate-buffered saline. Cell pellets were resuspended in NTN containing a cocktail of protease inhibitors (1 mM PMSF, 1 mg/ml pepstatin A, 5 mg/ml leupeptin and 5 mg/ml aprotinin), and incubated on ice for 20–30 min. Cell lysates were cleared by centrifugation at 18 000 *g* for 30 min at 4°C and then incubated with the appropriate resin [anti-flag agarose (Sigma); anti-HA agarose (Bethyl Inc.); nickel-sepharose (Pharmacia)] for 1–2 h at 4°C on a nutator. The beads were then washed four times with Low Salt Binding Buffer (20 mM sodium phosphate, pH 7.4 and 150 mM NaCl) (50 mM Tris, pH 7.9, 12.5 mM $MgCl_2$, 1 mM EDTA, 10% glycerol). Bound proteins were eluted by boiling the beads in SDS sample buffer and resolved on a SDS–polyacrylamide gel. Proteins were visualized by immunoblotting using the appropriate antibodies [anti-Flag M2 (Sigma), anti-HA, anti-myc (Bethyl Inc.)]. When indicated, the extract was incubated with 0.5 U/µl DNAse I (Invitrogen) at 25°C for 15 min before the immunoprecipitation reaction.

Exonuclease assay

Exonuclease activity was measured with the following DNA substrates: 20-oligomer A1 (5′-CGCTAGCAATATTCTGCAGC-3′), 20-oligomer A2 (5′-GCTGCAGAATATTGCTAGCG-3′) complementary to A1, and 46-oligomer A3 (5′-GCGCGGAAGCTTGGCTGCAGAATATTGCTAGCGGGAAATCGGCGCG-3′) partially complementary to A1. Oligonucleotides were labeled at the 5′ end with radiolabeled ATP. The appropriate oligonucleotides were annealed by boiling and slow cooling to room temperature. Reaction mixtures contained 40 mM Tris–HCl (pH 7.5), 4 mM $MgCl_2$, 5 mM DTT, 1 mM ATP, 0.1 mg/ml BSA, DNA substrates (∼40 fmol, 100 000 c.p.m.), and 50–200 fmol of atWEX protein, 100 fmol of WRN, 50–200 fmol of *at* Ku or *hs*Ku in a final volume of 10 µl. The reaction mixtures were incubated at room temperature for 10–20 min and then the reactions were terminated by the addition of 2 µl of a formamide-dye solution (95% formamide, 50 mM EDTA, 0.5% bromphenol blue and 0.5% xylene cyanol). After incubation at 95°C for 3 min, DNA products were resolved by either 12 or 16% polyacrylamide-urea gel electrophoresis and visualized by autoradiography.

Electrophoretic mobility shift assay

The 20mer (A1) oligonucleotide was labeled at the 5′ end with radiolabeled ATP and T4 polynucleotide kinase and then annealed to a partially complementary 46mer (A3). Radiolabeled oligonucleotide (80 fmol and 200 000 c.p.m.) was incubated with increasing amounts (100–400 fmol) of *at* Ku in 10 µl of buffer (10 mM Tris–HCl, pH 7.5, 80 mM NaCl, 4 mM KCl, 2 mM EDTA and 10% glycerol) at 25°C for 10 min. The samples were then resolved by electrophoresis through a 4% polyacrylamide gel at 10 V/cm in the cold room. The gels were dried on Whatman 3MM paper and subjected to autoradiography.

RESULTS

We wanted to examine *at* WEX exonuclease activity and its relationship to *hs*WRN and Ku. For this purpose, we isolated cDNAs for *at* WEX, *at* Ku70 and *at* Ku80 and cloned them in frame with a flag-epitope tag into baculovirus expression vectors. Each protein was expressed and purified from insect cells by affinity purification (Figure 2A). To demonstrate that they possess their respective activities, each recombinant protein was first tested in biochemical assays. Recombinant *at* Ku was examined by electrophoretic mobility shift assays for its ability to bind to DNA. As shown in Figure 2B, the addition of *at* Ku to a reaction mixture containing a radiolabeled double-stranded DNA (dsDNA) oligomer resulted in the formation of a strong protein–DNA complex, indicating that *at* Ku, like the homologous human factor (8), binds to linear dsDNA molecules. We then examined the exonuclease activity of recombinant *at* WEX. Incubation of *at* WEX with radiolabeled, dsDNA substrates produced strong hydrolysis of 3′ recessed ends (Figure 2C, lanes 2–4), and somewhat less efficient hydrolysis of 3′ overhang or blunt ends (Figure 2A, lanes 7–9, and Figure 2B, lanes 2–4). A mutant *at* WEX (encoded by the *wex*-2 allele) missing the conserved exonuclease domain III did not show any hydrolytic activity (Figure 2A, lanes 5 and 10, Figure 2B, lane 5), indicating that the observed activity

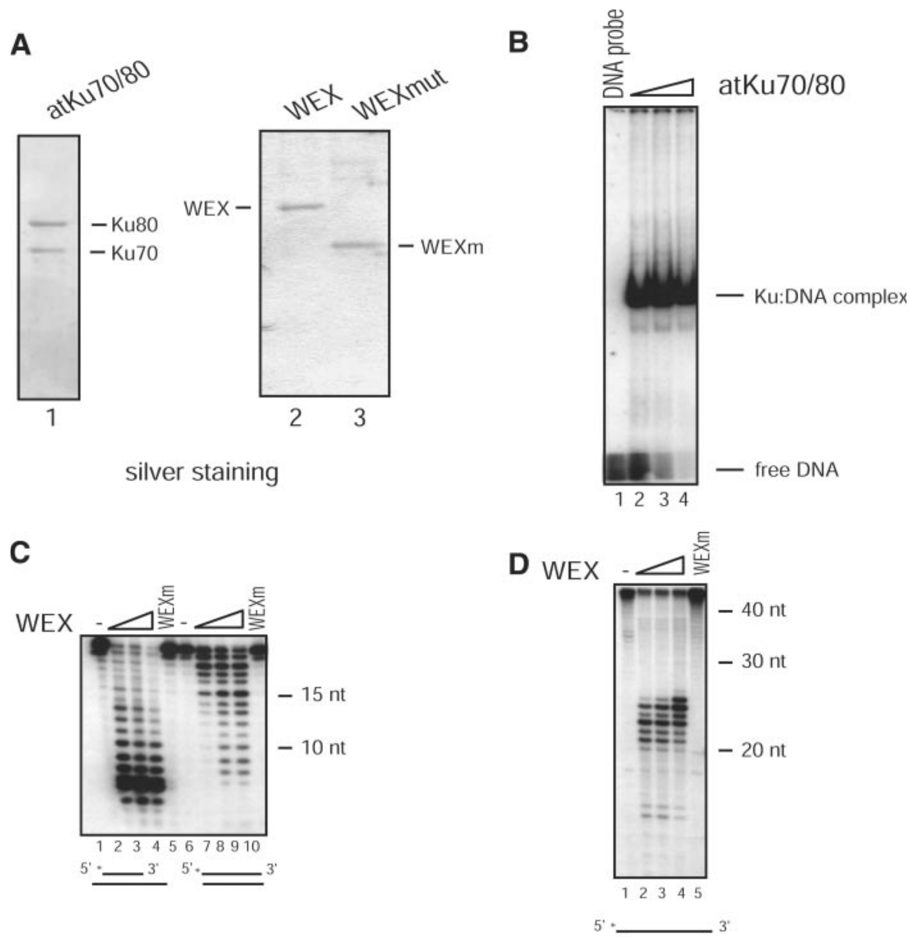

Figure 2. Activities of recombinant *at* WEX and *at* Ku. (**A**) Affinity-purified *at* Ku (lane 1), *at* WEX (lane 2) and mutant *at* WEXm (*wex-2*) (lane 3). Proteins were resolved by SDS–PAGE and visualized by silver staining. (**B**) *at* Ku binds to dsDNA. Radiolabeled 20mer (A1)/46mer (A3) DNA substrate was incubated with increasing amounts of *hs*Ku for 10 min at room temperature. The reactions were analyzed by 4% native PAGE, and the DNA–protein complexes were visualized by autoradiography (lanes 1, DNA probe only; lanes 2–4, 100, 200, 300 fmol of *at* Ku, respectively). (**C**) The 3′–5′ exonuclease activity of *at* WEX. Purified wild-type or mutant (*at* WEXm; W266*) *at* WEX were incubated with 3′ recessed, radiolabeled 20mer (A1)/46mer (A3) or blunt radiolabeled 20mer (A1)/20mer (A2) DNA substrate at room temperature for 20 min. Products were analyzed by 16% polyacrylamide-urea denaturing gel and autoradiography (lane 1, 20mer/46 mer probe only; lane 2–4, 50, 100 and 200 fmol of *at* WEX; lane 5, 100 fmol of *at* WEXm; lane 6, 20mer/20 mer probe only; lane 7–9, 50, 100 and 200 fmol of atWEX, respectively; lane 10, 100 fmol of *at* WEXm). (**D**) Purified *at* WEX and *at* WEXm were incubated with a radiolabeled 3′ protruding 46mer (A3)/20mer (A1) DNA substrate at room temperature for 20 min. Products were analyzed by 12% polyacrylamide-urea denaturing gel and autoradiography (lane 1, 46mer/20mer probe only; lanes 2–4, 400, 500 and 600 fmol of *at* WEX, respectively; lane 5, 500 fmol of *at* WEXm).

was intrinsic to *at* WEX and not caused by a co-purifying contaminant.

Next, we determined whether *at* WEX binds to *at* Ku. For this purpose, *Sf9* insect cells were infected with various combinations of recombinant baculoviruses expressing epitope-tagged *at* WEX, *at* Ku80 and *at* Ku70. Extracts from the infected cells were incubated with the appropriate antibody resin to capture flag-*at* WEX (Figure 3A) or myc-*at* Ku70 (Figure 3B), and the resulting immunoprecipitated products were resolved by SDS–PAGE. The presence of *at* Ku80 in the flag-*at* WEX immunoprecipitation reaction, or *at* WEX in the myc-*at* Ku70 immunoprecipitation reaction, was monitored by western blot with HA and flag antibodies, respectively. The results of these experiments indicate that both *at* Ku70 and *at* Ku80, as a complex or as single subunits, co-immunoprecipitated with *at* WEX. Reciprocal immunoprecipitation reactions confirmed that *at* WEX bound to Ku through interactions with both subunits (data not shown). The overall identity between *H.sapiens* and *Arabidopsis* Ku70 and 80 is 48 and 43%, respectively (BLASTP 2.2.10 analysis). Notwithstanding this limited sequence identity, *at* Wex also binds to *hs*Ku (Figure 3C), suggesting conservation in the interaction domains between these two factors. The possibility that this interaction was mediated by tethering of the proteins to DNA was discounted by treatment of the extracts with DNAseI (Figure 3C). In conclusion, these results indicate that *at* WEX, as observed with *hs*WRN, binds to Ku.

We next examined whether *at* Ku influences the exonuclease activity of *at* WEX. To this end, purified recombinant proteins were incubated with radiolabeled dsDNA substrates and the products of the reactions were examined by denaturing gel electrophoresis and autoradiography. As shown in Figure 4A and B (lanes 2–4), the addition of *at* Ku to the reaction mixture resulted in increased hydrolysis of 3′ recessed and blunt ended DNA substrates, indicating that *at* Ku stimulates the nucleolytic activity of *at* WEX, as shown previously for *hs*WRN and *hs*Ku (8). Stimulation of *at* WEX activity by *at* Ku was also observed on double-stranded oligomers with a 3′ overhang

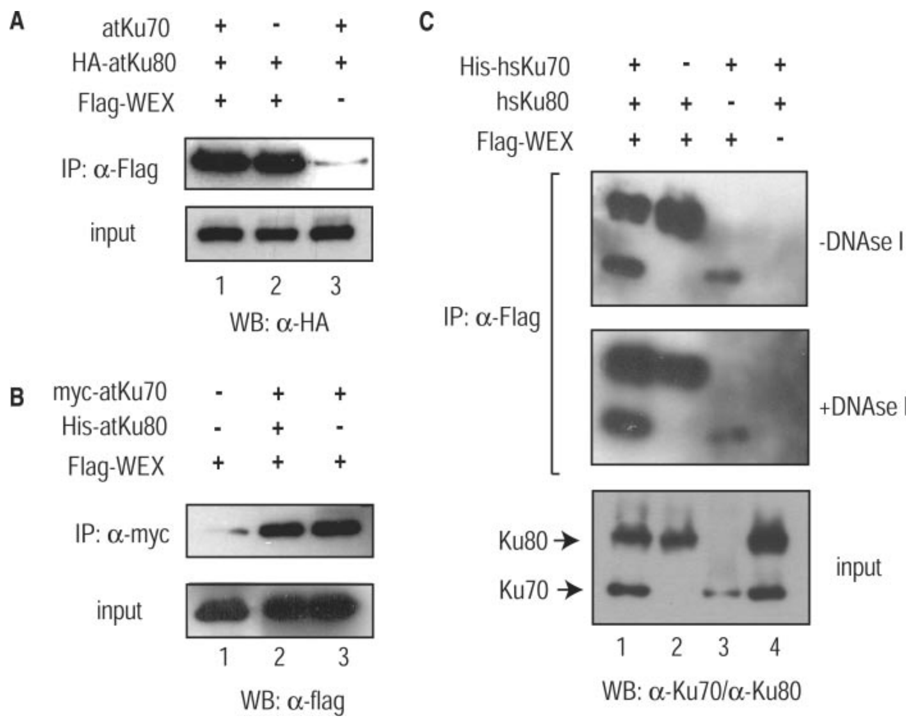

Figure 3. Physical interaction between *at* WEX and the Ku heterodimer. (**A**) *Sf9* cells were coinfected with baculoviruses expressing *at* Ku70, HA-*at* Ku80 and Flag-*at* WEX (lane 1), HA-atKu80 and Flag-*at* WEX (lane 2), and HA-*at* Ku80 and *at* Ku70 (lane 3). Cells were harvested 48 h postinfection, lysed in NTN buffer and the cleared lysates were incubated with anti-flag agarose resin for 2 h at 4°C on a nutator. The resin was washed extensively and then boiled in SDS sample buffer to release the bound proteins, which were separated by SDS–10% PAGE and transferred to nitrocellulose membrane for immunoblot analysis. Blotted *at* Ku80 was detected with HA antibody. The lower panel shows the expression level of *at* Ku80 in the cell extract from each coinfection. (**B**) Sf9 cells were infected with baculoviruses expressing Flag-*at* WEX (lane 1), myc-*at* Ku70, His-*at* Ku80 and Flag-*at* WEX (lane 2), or myc-*at* Ku70 and Flag-*at* WEX (lane 3). Cells were harvested 48 h postinfection, lysed in NTN buffer and the cleared lysates were incubated with anti-myc sepharose beads (Bethyl Inc.) for 2 h at 4°C on a nutator. Beads were then washed extensively and boiled in SDS sample buffer to release the bound proteins, which were separated by SDS–10% PAGE and transferred to nitrocellulose membrane for immunoblot analysis. *at* WEX was detected with Flag antibody (Sigma). The lower panel shows the expression level of *at* WEX in the protein extract from each coinfection. (**C**) Sf9 cells were infected with baculoviruses expressing His-*hs*Ku70, *hs*Ku80 and Flag-*at* WEX (lane 1), *hs*Ku80 and Flag-*at* WEX (lane 2), His-*hs*Ku70 and Flag-*atWEX* (lane 3), or hs*Ku80* and His-*hs*Ku70 alone (lane 4). Cells harvested 48 h postinfection were lysed in NTN buffer. Cleared lysates were divided into two aliquots and incubated at 25°C for 15 min in the absence (upper panel) or presence (lower panel) of DNase I (0.5 U/μl). Extracts were centrifuged briefly and incubated with anti-Flag sepharose beads for 2 h at 4°C on a nutator. Beads were then washed extensively and boiled in SDS sample buffer to release the bound proteins, separated by SDS–10% PAGE and transferred to nitrocellulose membrane for immunoblot analysis. The presence of *hs*Ku80 and *hs*Ku70 in the immunoprecipitation products was detected with anti-Ku70 and Ku80 antibodies (Santa Cruz Biotechnology). Lower panel shows the expression level of *hs*Ku80 and *hs*Ku70 in the protein extract from each coinfection.

(data not shown). There appears to be, however, a quantitative difference between *at* WEX and *hs*WRN exonuclease activity, with the latter displaying more dramatic activation by Ku.

We then examined whether the stimulation of *at* WEX and *hs*WRN exonuclease activities by Ku was conserved across species by assaying heterologous combinations of these proteins. Interestingly, while *at* Ku was very effective at stimulating *at* WEX exonuclease activity on both 3′ recessed and blunt DNA ends, *hs*Ku failed to significantly enhance *at* WEX exonuclease activity on both substrates (Figure 4A and B, lanes 5–7). Rather, stoichiometric amounts of *hs*Ku slightly inhibited *at* WEX exonuclease activity on substrates with a 3′ recessed DNA end (Figure 1A, compare lanes1 and 5), but not on DNA substrates with blunt ends. The addition of a 4-fold excess of *hs*Ku to *at* WEX, however, resulted in a minor but reproducible increase in exonuclease activity on the 3′ recessed ends (lane 7). The inability of Ku to stimulate the activity of the heterologous exonuclease was more dramatic in the reciprocal experiment, as *hs*WRN exonuclease activity was stimulated by *hs*Ku (lanes 9–11), but not by its *Arabidopsis* counterpart *at* Ku (lanes 12–14). Thus, a limited functional interaction was displayed by *at* WEX and *hs*Ku, but not by *hs*WEX and *at* Ku.

DISCUSSION

Rapid progress in genome analysis is leading to the identification of the genes responsible for many human diseases. Understanding the molecular basis of these inherited conditions, however, hinges on uncovering the function of the proteins encoded. Such an endeavor can be challenging, as in the case of the WS protein (WRN). WRN is a protein that may play an important role in human aging. Loss-of-function mutations in the WRN gene are associated with the premature onset of degenerative conditions generally observed during normal aging, such as cataracts, graying and loss of hair, atherosclerosis, loss of subcutaneous fat, diabetes, osteoporosis and certain types of cancer. WRN has been implicated in pathways such as DNA repair, recombination and replication, but the precise cellular function of WRN, and the molecular basis of WS, remains to be determined.

An important clue of function is provided by the identification of physical interactions between the protein of interest and other cellular factors. In previous studies we showed that the heterodimeric factor Ku forms a stable complex with WRN and stimulates its exonuclease activity. Ku is required for the repair of DNA DSBs by NHEJ (11), but has also been

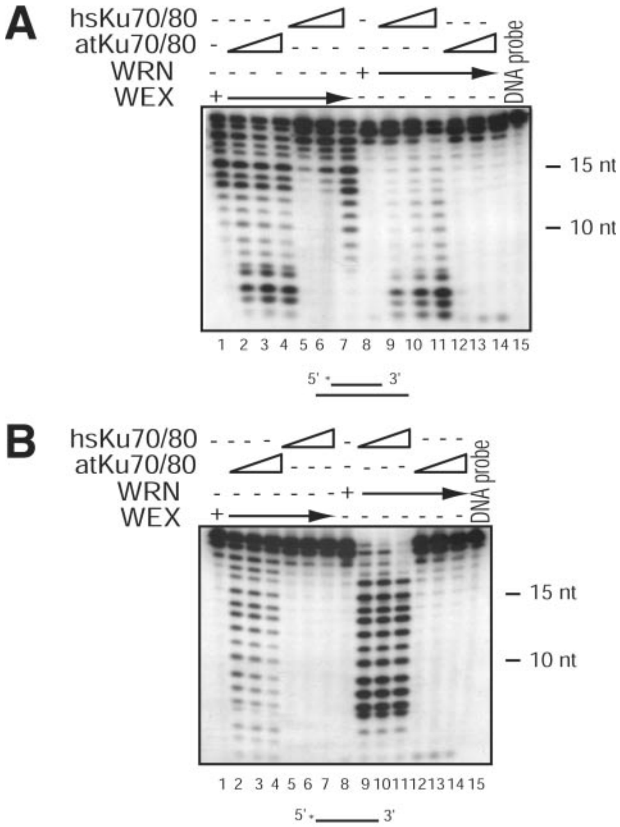

Figure 4. Species-specific stimulation of *at*WEX and *hs*WRN exonuclease activity by Ku. (**A**) Purified *at*WEX and *hs*WRN were incubated at room temperature for 10 min with radiolabeled 3′ recessed 20mer (A1)/46mer (A3) DNA substrate in the absence (lanes 1 and 8) or presence of *at*Ku (lanes 2–4 and 12–14) or *hs*Ku (lanes 5–7 and 9–11). Products were analyzed by 16% polyacrylamide-urea denaturing gel and autoradiography (lane 1, 50 fmol of *at*WEX; lanes 2–4, 50 fmol of *at*WEX and 50, 100 and 200 fmol of *at*Ku, respectively; lanes 5–7, 50 fmol of *at*WEX and 50, 100 and 200 fmol of *hs*Ku, respectively; lane 8, 100 fmol of *hs*WRN; lanes 9–11, 100 fmol of *hs*WRN and 50, 100 and 200 fmol of *hs*Ku, respectively; lanes 12–14, 100 fmol of *hs*WRN and 50, 100 and 200 fmol of *at*Ku, respectively; lane 15, DNA probe only). (**B**) Purified *at*WEX and *hs*WRN were incubated with radiolabeled blunt ended 20mer (A1)/20mer (A2) DNA substrate at room temperature for 10 min in the absence (lanes 1 and 8) or presence of *at*Ku (lanes 2–4 and 12–14) or *hs*Ku (lanes 5–7 and 9–11). Products were analyzed by 16% polyacrylamide-urea denaturing gel and autoradiography (lane 1, 50fmol of *at*WEX; lane 2–4, 50 fmol of *at*WEX and 50, 100 and 200 fmol of *at*Ku, respectively; lanes 5–7, 50 fmol of *at*WEX and 50, 100 and 200 fmol of *hs*Ku, respectively; lane 8, 100 fmol of *hs*WRN; lanes 9–11, 100 fmol of *hs*WRN and 50, 100 and 200 fmol of *hs*Ku, respectively; lanes 12–14, 100 fmol of *hs*WRN and 50, 100 and 200 fmol of *at*Ku, respectively; lane 15, DNA probe only).

implicated in the maintenance of telomere structure, acting as negative regulator of telomerase in animals (20) and plants (21). In plants, *at*Ku is required for proper maintenance of the telomeric C strand and regulates the extension of the telomeric G strand (22). In contrast to the mammal orthologous complex (20,23), however, *at*Ku is not required for fusion of critically short telomeres in telomerase-deficient *Arabidopsis*. Understanding the physiological role of the Ku–WRN interaction may provide key insights on WRN function. The evolutionary history of this interaction should help to elucidate its significance. *at*WEX is an *Arabidopsis* protein with homology to *H.sapiens* WRN. The similarity between *at*WEX and the N-terminal exonuclease domain of WRN prompted us to ask whether there was a functional relationship between these two factors. Conservation of the interaction across distant taxa would underscore its importance to the function of WRN. Our data show that recombinant *at*WEX has intrinsic exonuclease activity that hydrolyzes dsDNA oligomers with 3′ overhang, blunt and 3′ recessed ends. These results differ from a previous report, which showed that *at*WEX does not hydrolyze DNA with blunt ends (14). This discrepancy is probably owing to the different expression systems used for the isolation of recombinant proteins for the exonuclease assays [bacteria (14) versus insect cells (this study)]. The results of our studies also indicate that the exonuclease activity of *at*WEX only partially resembles the corresponding activity of *hs*WRN, which hydrolyzes dsDNA oligomers with a 3′ recessed end and is inactive on DNA with a 3′ overhang or blunt ends (8). Although *at*WEX displays broader substrate specificity, we cannot rule out that this difference results from the presence of the RecQ helicase and other domains within the C-terminal region of *hs*WRN, as these may influence the exonuclease activity of *hs*WRN. Indeed, a mutant *hs*WRN comprising only the exonuclease domain, *hs*WRN(1-388), displays stronger exonuclease activity than full-length *hs*WRN [B. Li and Lucio Comai, unpublished data and (10)]. When we tested whether Ku influences the activity of *at*WEX, we observed that atWEX exonuclease was strongly stimulated by *at*Ku, but only to a minor degree by *hs*Ku. This species-specific stimulation was also observed in the complementary experiment, which shows that *at*Ku could not augment *hs*WRN exonuclease activity. The inability of WRN-like exonucleases to be efficiently stimulated by heterologous Ku proteins indicates that Ku stimulation is not the result of a topological constraint on the substrate resulting from the binding of Ku to DNA. Rather, binding of *hs*WRN orthologs to Ku proteins and stimulation of exonuclease activity is a specific regulatory interaction that predates the split between plants and animals, an event ∼1.5 billion years old, and has been maintained through multiple evolutionary changes of these proteins. Divergence of the functional interaction may indicate that the WRN/WEX–Ku complex has evolved within each species to optimize function. We propose, therefore, that the relationship between WRN exonuclease and Ku is highly significant and that it is informative in determining the function of *hs*WRN and related proteins.

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Conflict of interest statement. None declared.

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